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Serogroup identification and Virulence gene characterization of *Listeria monocytogenes* isolated from chicken carcasses

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Abstract

Listeria monocytogenes is an important food borne pathogen with a high case fatality rate. Among 13 different serotypes of this bacterium, 4 serotypes (1/2a, 1/2b, 1/2c, and 4b) were considered as the main cause of listeriosis outbreaks. The aim of this study is to identify the major serotypes and virulence genes in Listeria monocytegenes isolated from chicken carcasses which were collected from different supermarkets and butcheries in Mashhad. Among the 80 isolated *Listeria* spp., most of them were identified as *L. monocytogenes* (36 out of 80). Most of the *L. monocytogenes* isolates belonged to serogroup llb (52.77%) which contains 1/2b and 3b serotypes. The second and third major serogroups were IVa (27.77%) and IIa (16.66%). Serogroup IVb (2.77%) which contains 4b serotype was the fourth major isolate. In order to differentiate serotype 1/2a and 3a from 1/2c, amplification of *flaA* gene was used. *L. monocytogenesis isolates were* also examined for the presence of *inlC*, *inlJ* and *hlyA* virulence genes. 26 out of 36 isolates were positive for *inlC*, and inlJ, whereas hlyA gene was detected in 32 isolates. Chicken carcasses may act as a source of infectious listeriosis for humans living in this area.

Keywords: *L. monocytogenes*, Serotype, inlC, inlJ, hlyA.

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Introduction

Listeria monocytogenes is an intracellular gram positive pathogen that is widely distributed in nature and contaminates different kinds of food. Refrigerated ready to eat food is of the most concern due to ability of multiplication of *Listeria* under refrigeration temperature (Liu *et al.*, 2006). Susceptible populations toward food born listeriosis are pregnant women, neonates, the elderly, and immunosuppressed individuals. Disease primarily causes abortion, septicaemia or infections of the central nervous system (Rossi *et al.*, 2008).

L. Monocytogenes are classified into 13 different serotypes based on somatic (O) and flagellar (H) antigens. 1/2a, 1/2b, 1/2c, and 4b are the four main pathogenic serotypes from which 1/2a, 1/2b and 4b are responsible for 98% of human listeriosis. 4a and 4c are the other serotypes which are rarely associated with outbreaks of the disease (Wiedmann *et al.*, 1996; Jacquet *et al.*, 2002). Recently, a multiplex PCR assay has been proposed by Doumith *et al.*, 2004 for the identification of four major serovars of *L . monocytogenes* strains into distinct serogroups.

Doumith *et al.*, 2004, targeted four marker genes in multiplex PCR assay. They identified lmo0737 and lmo1118, in the sequence of *L. Monocytogenes* serovar 1/2a, and ORF2819 and ORF2110, in the sequence of *L. monocytogenes* 4b strain (Doumith *et al.*, 2004). Borucki and Call, 2003, designed a PCR for further differentiation of serotypes 1/2a and 3a from 1/2c and 3c by the amplification of *flaA* gene, which encodes the *L. Monocytogenes* flagellin Protein. Strains which resulted positive with the FlaA primers were considered as serotype 1/2a or 3a whereas negative strains were considered as serotype 1/2c or 3c.

However, diverse *L. monocytogenes* strains varied in pathogenicity and virulence (Liu *et al.*, 2003a). Adherence of *L. monocytogenes* to host cells and their internalization takes place by the assistance of a family of surface proteins called internalins, especially *InlA* and *InlB*. Besides *InlA* and *InlB*, other internalins such as *InlC* and *InlJ* also appear to be contributing in the later (i.e., post intestinal) stages of *L. monocytogenes* infection (Engelbrecht *et al.*, 1996; Gouin *et al.*, 1994). Different genes were considered for identification of virulence strains, some of which were directly related to virulence of *L. monocytogenes* (Sabet *et al.*, 2005). However, some of them were associated with virulence associated protein. Liu *et al.*, 2007, proposed a PCR assay for rapid and sensitive identification of virulent strain of *L. monocytogenes*. These genes included *inlC* (lmo1786) and *inlJ* (lmo2821) encoding putative internalins. *inlJ* (lmo2821) is present in strains of *L. monocytogenes* that are able to cause human outbreak (Liu *et al.*, 2004a). The target of choice for rapid laboratory differentiation of virulent from avirulent *L. monocytogenes* strains is lmo2821 (i.e. inlJ) (Liu, 2006).

Another virulence factor that is very important in *L. monocytogenes* virulence is listeriolysin O (LLO). LLO (a 58 KDa protein encoded by *hlyA* gene) is a pore-forming toxin that is essential for virulence of *L. monocytogenes* (Vazquez-Boland *et al.*, 2001). Listeriolysin O (LLO) is a protein encoded by *hlyA* gene. This protein lyses the membrane of the vacuole and finally assists to the escape of *L. monocytogenes* from the vacuole into the cell (Vazquez-Boland *et al.*, 2001).

The aim of the present study is to determine the major serotype of *L. monocytogenes* in chicken carcasses and determine the major pathotype of this pathogen in the Northeast of Iran.

Materials and methods

Sampling

A total of 200 fresh chicken carcasses were collected randomly from different supermarkets and butcheries in Mashhad. Each Chicken carcass was placed in a sterile, large plastic bag with 250 ml sterile distilled water and was massaged for 1 minute inside the bag.

After removing the Chicken carcass, the plastic bags containing the rinsed fluid were immediately transported to the laboratory inside a portable ice-chest and bacterial analysis was started within 1-4 hours (Faverom *et al.* 1968).

Isolation and Identification of bacteria

After filtration with sterilized cheese cloth and centrifugation at 4000 rpm for 10 minutes of each rinsed fluid in four 50 ml falcon tubes, the supernatant fluid was decanted. The pellets obtained by centrifugation were unified and resuspended in 9 ml of listeria enrichment broth (LEB, Merck, Germany) containing 15 mg/l acriflavine, 40 mg/l nalidixic acid and 50 mg/l cycloheximide. All samples were incubated at 30 °C for 48 h. After incubation, 0.1 ml of the enriched culture was spread on Oxford agar plate supplemented with Natamycin 25 mg/l, Colistin sulphate 20 mg/l, Acriflavine 5 mg/l, Cefotetan 2 mg/l, and Fosomycin 10 mg/l (Listeria Selectival-SV33 Series-Mast Diagnostic, Germany) which was incubated at 30 °C for 48 h (Hitchins & Jinneman, 2013). Colonies that hydrolyzed aesculin were streaked onto another plate and were incubated at 30 °C for 24 h. Gram positive bacilli that were catalase positive and displayed tumbling motility at room temperature and umbrella motility at 30 °C were considered for DNA extraction.

M-PCR confirmation of Listeria monocytogenes

DNA extraction was done using the boiling method (Amagliani *et al.*, 2007). Two pairs of primers were used for confirmation of *Listeria spp.* and *Listeria monocytogenes* using multiplex PCR assay. The *prs* primers are specific for the putative phosphoribosil pyrophosphate synthetase gene of *Listeria* spp. and the *LM lip1* primers are specific for the *prfA* gene of *Listeria monocytogenes.* The sequences of the primers which were used and the predicted sizes of the amplified products are shown in Table 1.

Amplification of bacterial DNA was performed using Cinna-Gen PCR Mastermix (25 µl volumes) (Sinaclon, Iran). Every reaction contained 7 and 10 (0.7 and 1µl) picomol of *prs* and *LM lip1* primers, respectively, and 2.5 µl of the DNA template, and 6.6 µl deionized distilled water. PCR reaction was performed in a thermal cycler (Techne, Germany). The PCR condition was carried out as follows: 94°C for 5 min for initial denaturation followed by 35 cycles of 94°C for 45 seconds, 54 °C for 30 seconds, and 72°C for 1 min and a final extension of 72°C for 10 min.

Listeria monocytogenes (ATCC 7644) and deionized distilled water were used as positive control and negative control, respectively.

Multiplex-PCR for serogroup identification

A multiplex-PCR assay was carried out to separate the major *L. Monocytogenes* serovars (1 ⁄ 2a, 1 ⁄ 2b, 1 ⁄ 2c and 4b) into distinct serogroups. The marker genes were selected, according to the previous studies (Borucki and Call, 2003; Doumith *et al*. 2004), which were *lmo0737*, *lmo1118*, *ORF2819*, O*RF2110*, and *FlaA*. The *prs* gene, specific for *Listeria spp.* or *prfA* gene of *Listeria monocytogenes*, was used as an internal amplification control. Target genes, primer sequences, specificity and PCR products size are listed in Table 1. Briefly, 4µl (40 nanogram) of extracted DNA was used for the amplification; each reaction was performed in a 25µl final volume containing 12.5µl of PCR Master Mix 2X (Ampliqon, Denmark) and five primer sets. Primers concentrations were as follows: 1.2 µl (12 picomol) of *lmo0737*, 1.2 µl (12 picomol) *lmo1118*, 1.6 µl (16 picomol) *ORF2819*, 1.6 µl (16 picomol) *ORF2110 a*nd 0.4 (4 picomol) µl *prs*. Amplification conditions were carried out as follows: 94°C for 3 min for initial denaturation, followed by 35 cycles of 94°C for 40 seconds, 55 °C for 45 seconds, and 72°C for 1 min and a final extension of 72°C for 7 min.

Reference strains ATCC 7644, and IBRC 10671 (which is equivalent to strain ATCC 13932) covering the main serotypes, 1/2c and

4b respectively, were used as positive controls for each amplification assay and deionized distilled water was used as negative control.

A second PCR assay was performed to detect the presence of the *flaA* gene encoding the *L. Monocytogenes* flagellar protein (specific for serotype 1/2a and 3a) according to the protocol described by Borucki and Call (2003). The amplification mix consisted of 25 µl, with 12.5 µl of 2X PCR Mastermix, 1.6 µl of *flaA*-F and *flaA*-R primers and 0.8 µl of *prs* primers (Table 1). Amount of 2 µl of DNA template was used. The PCR conditions were as follows: initial denaturation for 3 min at 94 °C, followed by 40 cycles of denaturation at 94 °C (30 s), annealing at 61 °C (40 s), extension at 72 °C (1 min) and a final extension at 72 °C (7 min).

Four microlitres of the PCR products were separated on a 1.5% agarose gel (Merck, Germany) and were visualized on a transilluminator after ethidium bromide staining.

Detection of Virulence genes

A multiplex PCR assay was used for detection of *inLC* and *InlJ* virulence genes of *Listeria monocytogenes* isolates. Every reaction contained 12.5 µl of 2X PCR mastermix, 1 µl of each oligonucleotide primers (Table 1), 2 µl of the DNA template, and 6.5 µl of deionized distilled water. The PCR condition was carried out as follows: 94°C for 2 min for initial denaturation, followed by 35 cycles of 94°C for 20 seconds, 55 °C for 20 seconds, and 72°C for 50 seconds and a final extension of 72°C for 2 min.

Another PCR assay was performed in order to detect *hlyA* gene, encoding listeriolysin, O (LLO) toxin of *Listeria monocytogenes* isolates using specific primers (Table 1). In each reaction 12.5 µl of 2X PCR mastermix, 1 µl of hlyA primer and 1 µl of the DNA template were used. The PCR condition was carried out as follows: 95°C for 2 min for initial denaturation, followed by 35 cycles of 95°C for 15 seconds, 60 °C for 30 seconds, and 72°C for 60 seconds and a final extension of 72°C for 10 min.

Results

Occurrence of Listeria spp. and L. Monocytogenes in chicken carcasses

In the present study, out of the 200 samples of fresh chicken carcasses, *Listeria spp.* was detected in 80 (40%) of the samples. Using conventional culture method, *Listeria spp.* was identified in 80 samples, which was confirmed in m-PCR assay. Most of the *Listeria* isolates were determined to be *L. monocytogenes* (36 out of 80) (Fig. 1).

Serogroup identification

The extracted DNA from all 36 isolates of the *L. monocytogenes* which were confirmed by amplification, the 370 bp of *prs* and 274 bp of *prfA* genes, were used for serogroup identification. The multiplex PCR allowed *L. monocytogenes* strains to be clustered into five molecular serogroups: IIa corresponded to the presence of *lmo0737* gene (*L. Monocytogenes* serovars 1/2a, 1/2c, 3a, and 3c); IIb corresponded to the presence of *ORF2819* gene (*L. Monocytogenes* serovars 1/2b, 3b, 4b, 4d, and 4e); IIc corresponded to the presence of *lmo0737* and *lmo1118* genes (*L.* Monocytogenes serovars 1/2c and 3c); and IVb corresponded to the presence of *ORF2819* and *ORF2110* genes (*L. Monocytogenes* serovars 4b, 4d, and 4e). The fifth serogroup, IVa (*L. Monocytogenes* serovars 4b, 4d, and 4e), corresponded to the *L. monocytogenes* isolates for which none of the four tested genes could be detected.

Most of the isolates belonged to IIb group (52.77%) that contains 1/2b and 3b serotypes. Serogroups IVa (27.77%) and IIa (16.66%) were the second and the third major groups. Serogroup IVb (2.77%) containing the 4b strain was the fourth major group. In order to differentiate serotypes 1/2a and 3a from 1/2c, amplification of *flaA* was used. None of the isolates were positive for this gene. In fact, these isolates were identified as 1/2c or 3c. Table 2 shows the presence of four marker genes of the serogroups in the isolates of *L. monocytogenes*.

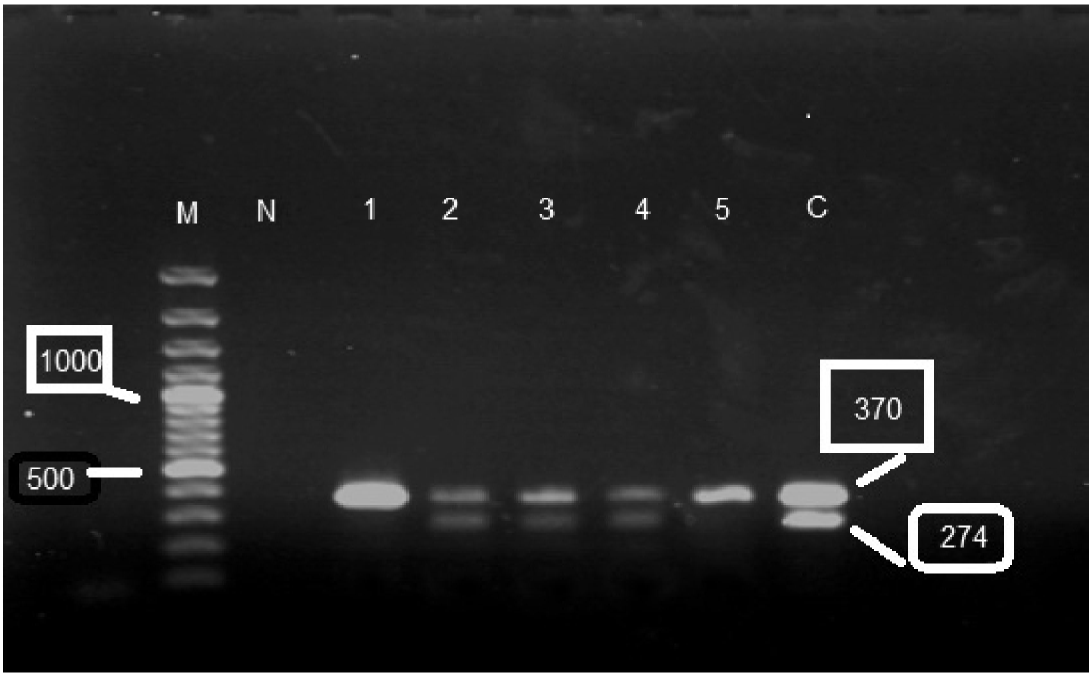


Figure 1. m-PCR detection of *L. monocytogenes*. Lane M: DNA Size Marker (100bp plus); Lane N: Negative Control; Lane C: Positive control; Lane 1 & 5: Positive samples for *Listeria spp* other than *monocytogenes*; Lane 2, 3 & 4: Positive samples for *Listeria monocytogenes*.

Table 1. Sequence and specificity of primers and the size of the amplified products

Target Gene	Sequence (5´-3´)	Specificity	Reference	Pcr Product Size (Bp)
prs	GCT GAA GAG ATT GCG AAA GAA G CAA AGA AAC CTT GGA TTT GCG G	All *Listeria spp.*	Doumith *et al*. (2004)	370
LM LIP1	GAT ACA GAA ACA TCG GTT GGC GTG TAA TCT TGA TGC CAT CAG	*Listeria monocytogenes*	Wernars *et al*., (1992)	274
lmo0737	AGGGCTTCAAGGACTTACCC ACGATTTCTGCTTGCCATTC	*L. monocytogenes*serovars 1/2a, 1/2c, 3a, and 3c	Doumith *et al*. (2004)	691
lmo1118	AGGGGTCTTAAATCCTGGAA CGGCTTGTTCGGCATACTTA	*L. monocytogenes*serovars 1/2c and 3c	Doumith *et al*. (2004)	901
ORF2819	AGCAAAATGCCAAAACTCGT CATCACTAAAGCCTCCCATTG	*L. monocytogenes*serovars 1/2b, 3b, 4b, 4d, and 4e	Doumith *et al*. (2004)	457
ORF2110	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGAC	*L. monocytogenes*serovars 4b, 4d, and 4e	Doumith *et al*. (2004)	597
FLAA	TTACTAGATCAAACTGCTCCC AAGAAAAGCCCCTCGTCC	Serotypes 1/2a and 3a	Borucki and Call (2003)	538
INL C	AATTCCCACAGGACACAACC CGGGAATGCAATTTTTCACTA	-	Liu *et al*., (2007)	517
INL J	TGTAACCCCGCTTACACAGTT AGCGGCTTGGCAGTCTAATA	-	Liu *et al*., (2007)	238
HLYA	GCAGTTGCAAGCGCTTGGAGTGAA GCAACGTATCCTCCAGAGTGATCG	-	Doijad *et al*., (2011)	456

Table 2. Serogroups of *L. monocytogenes* isolates according to the presence of four marker genes by using m-PCR assay.

Species	No of isolates (%)	m-PCR fragment amplification					Serovar group classification
		lmo0737 (691 bp)	lmo1118 (906 bp)	ORF2819 (471 bp)	ORF2110 (597 bp)	Prs(370 bp)	
L. monocytogenes	19 (52.77%)	-	-	+	-	+	IIb, serovars 1/2b, 3b, 7
L. monocytogenes	6 (16.66%)	+	-	-	-	+	IIa, serovars 1/2a, 1/2c, 3a, and 3c
L. monocytogenes	10 (27.77%)	-	-	-	-	+	IVa, serovars 4a, and 4c
L. monocytogenes	1 (2.77%)	-	-	+	+	+	IVb, serovars 4b, 4d, and 4e

Virulence characterization of L. monocytogenes isolates

In this study, 36 isolates of *L. monocytogenes* were examined for the presence of *inl C*, *inlJ* and *hly* virulence genes. *InLC*, and *InlJ* genes were detected in 26 isolates, whereas the hlyA gene was detected in 32 isolates. Table 3 shows the details of the presence of virulence genes in the isolates.

Table 3. Presence of virulence genes in *L. monocytogenes* isolates using PCR primers

Number of isolates	*inl C*	*inl J*	*hly*
4	-	-	-
26	+	+	+
6	-	-	+

Discussion

For a long time, *L. Monocytogenes* have been regarded as animal pathogens. In the late 1970s and early 1980s, *L . monocytogenes* have emerged as a significant food borne pathogen after the increasing consumption of RTE food products (Goulet, 2006).

In the present study, *Listeria spp*. was detected in 40% of fresh chicken carcasses. In another study the prevalence of *Listeria* in chicken carcasses has been reported to be 40.7% in Iran (Fallah *et al*., 2012). In our study *Listeria monocytogenes* were predominant among the isolated *Listeria spp.* and were detected in 18% of fresh chicken carcasses. Fallah *et al.* (2012) reported *L. Monocytogenes* in 14.1% of raw poultry products. In other studies, the rate of contamination with *L. monocytogenes* in raw poultry products was found to be 41% in Portugal (Antunes *et al*., 2002) and 38% in Northern Greece (Sakaridis *et al*., 2011), which are higher than the results of the present study.

In the present study, most of the isolates belong to serogroup IIb (52.77%) that mostly contains 1/2b and 3b serotypes These serotypes were also the most common serotype in poultry products in USA (Zhang *et al*., 2007). Fallah *et al. (* 2012) in Isfahan province of Iran reported the prevalence of serotype 1/2b of *L. monocytogenes* isolates in poultry products as (10.2%), whereas 4b serotype was the predominant serotype in their study (44.9%). However, in our study, serogroup IVb (2.77%) containing 4b, 4d and 4e strains was the fourth major serogroup. The "4b, 4d, 4e" serogroup prevalence for the 32 *L. monocytogenes* from RTE foods in Malaysia was reported as 12.5% (Jamali & Tthong, 2014). The 1/2a, 1/2b, and 4b serovars are mainly associated with most cases of human listeriosis. Although serovar 4b is commonly associated with human listeriosis (50% - 70%), it is not the most common serotype isolated from food (Martins & Leal Germano, 2011).

Serogroup IIa containing 1/2a, 3a, 1/2c and 3c serotypes was the third major serogroup occurring in 16.66% of the samples. In Portuguese and Estonian, Serotype 1/2a was predominant in poultry products (Guerra *et al.*, 2001; Praakle-Amin *et al*., 2006). Fallah et *al.*

(2012) reported the prevalence of 1/2a serotype as 40.8%, and 1/2c serotype as 4.08% in poultry products in another area of Iran. In Finland, serotype 1/2c was found to be the most common serotype in poultry products (Miettinen *et al*., 2001), although, they identified serotypes by agglutination method that may be the cause of different results in their studies. In RTE foods, the serogroup distribution of 1/2a, 3a and 1/2c, 3c was determined to be 65.6% and 21.9%, respectively (Jamali & Tthong, 2014).

In the present study, IVA (27.77%) was the second major serogroup. This serogroup contains 4a and 4c serotypes that are mostly nonpathogenic to humans and rarely associated with the disease. In a study by Pagadala *et al*., (2012) in USA, a large group (73.3%) of *L. monocytogenes* isolates from blue crab belonged to serotypes 1/2a, 1/2b and 4b. Doijad *et al*., (2011) revealed that all the 16 isolates of *L. Monocytogenes* which were isolated from poultry products belonged to serogroup IIa. According to Sant'ana *et al*., (2012), most pathogenic *L. monocytogenes* isolates that belonged to serotypes 4b and 1/2b and 97% of the isolates were positive for *inlC* and *inlJ* genes.

Cabrita *et al*., (2010) evaluated the secretome of two virulent and avirulent strains of *L. monocytogenes*. They showed that LLO was consistently present in the secretomes of 1/2a, 4b and 4c serovars. However, *inlC* was only present in the secretome of virulent strains (serovar 4b and 1/2a) and was absent in low virulent strains (serovar 4d/4e/4c) (Cabrita *et al*., 2010).

In this study, all the *L. monocytogenes* isolates were subjected to PCR detection of the *inlC*, *inlJ* and *hlyA* genes. All *L. monocytogenes* isolates which belonged to IIa, IIb and IVb serogroups were positive for these genes. Totally 75% of the isolates were positive for *inlC* and *inlJ* genes. In the previous studies, *inlC* and *inlJ* genes were positive in all the examined *L. monocytogenes* isolates which were obtained from the environment and RTE food samples (Gelbícová & Karpísková, 2012; Jamali & Tthong, 2014).

Liu *et al*., 2007 showed that the presence of *inlC* and *inlJ* genes in *L. monocytogenes* strains could increase mortality in mice via the intraperitoneal route. Therefore, *L. monocytogenes* isolates containing these genes have a great potential to cause human infections (Liu *et al*., 2007).

Jamali *et al*. (2013) and Mammina *et al*. (2009) reported the presence of *inlC* and *inlJ* in *L. monocytogenes* isolates from raw milk and humans, respectively.

In this study, 90% of the isolates were positive for *hlyA* gene, which encodes the LLO. Our results are similar to the previous study in which all the *L. monocytogenes* isolates were positive for *hlyA* gene (Doijad *et al*., 2011). The *hlyA* gene has an important role in the invasion process of *L. monocytogenes*. However, isolates which contained some mutated form of this gene, do not have any pathogenicity. In other words, they are unable to cause any infections (Vazquez-Boland *et al*., 2001).

This is the first study in Iran in which the authors determine the serotype of *L. monocytogenes* using the molecular method and their virulence characterization. The results described here indicate that most of the isolates were grouped in 1.2b and 3b serogroups which are the main cause of human listeriosis. Also, most of these isolates contained most important virulence genes. Chicken carcasses may act as vehicles of transmission of virulent *L. monocytogenes* to humans, and declaring the potential health hazard of this when consumed by immuno compromised persons. It is hoped that in the future studies, the status of *L. Monocytogenes* in human will be evaluated.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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سروتایپینگ و شناسایی ژنهای حدت در جدایه‌های لیستریا مونوسیتوژنز لاشه مرغ

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چکیده

لیستریا مونوسیتوژنز یکی از پاتوژن‌های مهم منتقله از طریق مواد غذایی با میزان مرگ ومیر بالای افراد بیمار می‌باشد. در بین سیزده سروتیپ مختلف این باکتری، سروتیپ‌های 1/2a, 1/2b, 1/2c و 4b لیستریا مونوسیتوژنز مسئول اغلب موارد انسانی لیستریوز می‌باشند. هدف از مطالعه حاضر، شناسایی سروتیپ‌های غالب و ژن‌های حدت در باکتری‌های لیستریا مونوسیتوژنز جدا شده از لاشه‌های مرغ جمع‌آوری شده از سطح شهر مشهد می‌باشد. در بین 80 باکتری جدا شده لیستریا، اکثریت آنها متعلق به گونه لیستریا مونوسیتوژنز (45%) بودند. اغلب جدایه‌های *لیستریا مونوسیتوژنز*(52/77%) متعلق به گروه سروتیپی IIb (سروتیپ‌های 1.2b و 3b) بودند. دومین و سومین گروه سروتیپی غالب، IVaو IIaبه ترتیب با فراوانی 27/77% و 16/66%بودند. گروه سروتیپی IVb (2/77%) حاوی سروتیپ 4bچهارمین گروه غالب می‌باشد.جهت تمایز سروتیپ‌های 1/2a و 3a از 1/2c، از تکثیر ژن *flaA*استفاده گردید. جدایه‌های *لیستریا مونوسیتوژنز* از نظر ژن‌های حدت*inlC*، *inlJ* و *hlyA*مورد ارزیابی قرار گرفتند. دو ژن *inlC* و *inlJ* در 26 جدایه و *hlyA*در 32 جدایه لیستریا مونوسیتوژنز شناسایی گردید. با توجه به نتایج حاصله از این مطالعه گوشت مرغ میتواند به عنوان یکی از منابع عفونت لیستریوز در انسان در این منطقه مطرح باشد.

واژگان کلیدی: *لیستریا مونوسیتوژنز*، سروتیپ، inlC، inlJ، hlyA.